2009

Isolation of a 3-Methylindole-Producing Bacterium from a Swine Waste Lagoon

Michelle L. Fusting
Western Kentucky University

ISOLATION OF A 3-METHYLINDOLE-PRODUCING BACTERIUM FROM A SWINE WASTE LAGOON

by

MICHELLE L FUSTING

Under the Direction of Kinchel Doerner

ABSTRACT

With an increase in animal production closer to populated areas, odorants from animal waste are a rapidly rising concern. 3-Methylindole (3-MI) is an odorant produced from the biodegradation of L-tryptophan by bacteria in animal waste. Currently, no 3-MI producing bacterium has been isolated from swine waste, and isolation of such an organism would provide insight into production and regulation of 3-MI. Therefore, an experiment was designed to isolate a 3-MI producer based on previous work which indicates the presence of Fe(III) increases 3-MI levels. An *Enterococcus sp.* was isolated from WKU's primary swine waste lagoon in rich, anaerobic medium plus tryptophan and Fe(III). Successive laboratory culturing of this bacterium resulted in the loss of 3-MI

production, which may be due to a lack of nutritional requirements. Attempts to restart 3-MI production by supplementing different nutritional components to the media were unsuccessful. Current results indicate this strain does not produce 3-MI.

INDEX WORDS: Malodorant, 3-Methylindole (3-MI), Skatole, Swine waste lagoon, Bacterium isolation, Anaerobic conditions

ISOLATION OF A 3-METHYLINDOLE-PRODUCING BACTERIUM FROM A SWINE WASTE LAGOON

by

MICHELLE L FUSTING

A Capstone Experience/Thesis

submitted in partial fulfillment of the requirements of

University Honors College at

Western Kentucky University

2009

ISOLATION OF A 3-METHYLINDOLE-PRODUCING BACTERIUM FROM A SWINE WASTE LAGOON

by

MICHELLE L FUSTING

Committee Chair: Kinchel C Doerner

Committee: Rodney A King
Kevin M Williams
Baozhen Luo

Electronic Version Approved:

Honors College
Western Kentucky University
May 2009

ACKNOWLEDGEMENTS

The author would like to thank the following people and groups for their contributions and assistance to this project: Dr. Kinchel Doerner, Donna Kridelbaugh, Bryan Mason, WKU Biotech Center – ARTP, WKU Biology Dept, WKU Honors College, USDA-ARS (USDA #58-6406-1-017).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

Rapid growth in human population has led to an increase in animal production. This has caused a rise in animal odorants in increasingly condensed areas. Malodorants from these food production animals are both a public nuisance and a health concern (Mackie *et al.* 1998). 3-Methylindole (3-MI), or skatole, is a prominent odorous compound produced by anaerobic bacteria in animal waste via the process of decomposition. 3-Methylindole has been shown to cause boar taint in pigs and acute bovine pulmonary emphysema in cattle (Deslandes *et al.* 2001). Several 3-MI producers have been isolated from environments such as food (Holdeman *et al.* 1977) and bovine rumen fluid (Yokoyama *et al.* 1977), although none have been isolated from animal waste. Studies of these 3-MI-producing organisms have shown that the amino acid, L-tryptophan, is the precursor to 3-MI. Tryptophan undergoes a transaminase reaction followed by one decarboxylation reaction to produce the intermediate indoleacetic acid, then undergoes another decarboxylation reaction to yield 3-MI (Figure 1; Chung *et al.* 1975, Whitehead *et al.* 2008). The majority of 3-MI producers can only generate 3-MI from the intermediate, indoleacetic acid (Deslandes *et al.* 2001); for example, *Lactobacillus* spp., isolated from the rumen, cannot directly utilize tryptophan to produce 3-MI, but instead use indoleacetic acid (Yokoyama *et al*. 1977, Yokoyama *et al.* 1981). Of known 3-MI producing bacteria, *Clostridium scatalogenes* is the only isolate identified to produce 3-MI from degradation pathway precursor, tryptophan (Doerner *et al*. 2008, Whitehead *et al.* 2008).

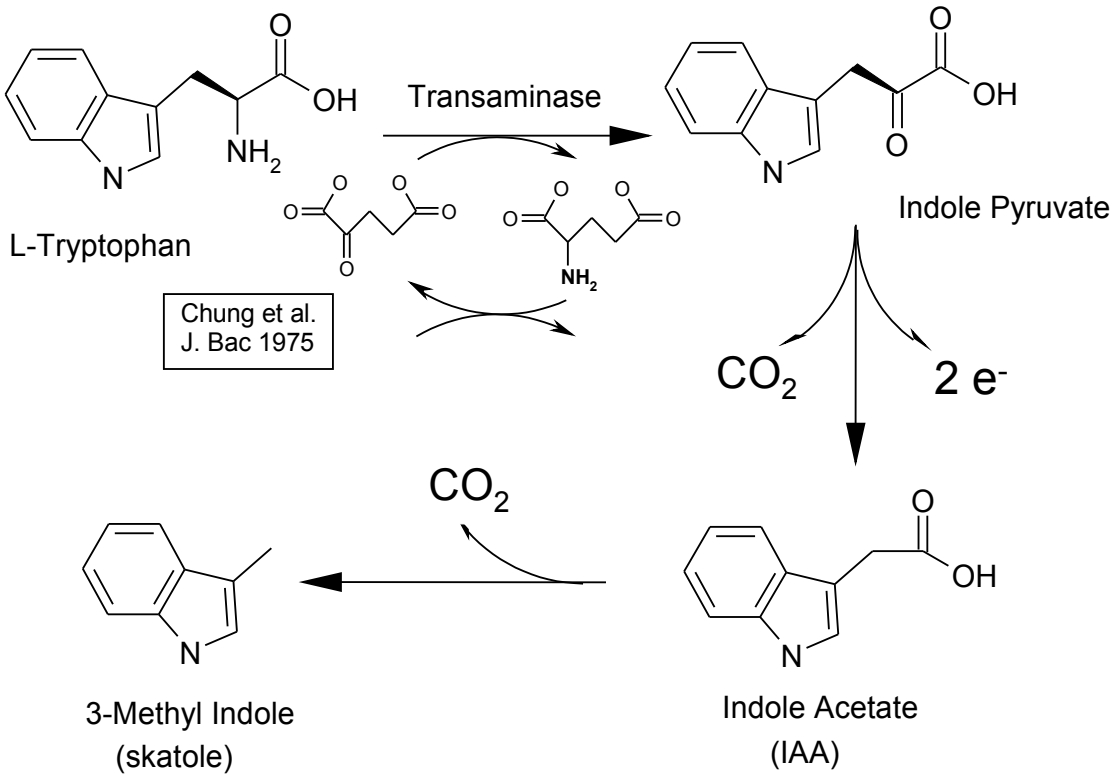

Figure 1. The biodegradation of L-tryptophan to produce the compound, 3-MI.

Little is known about the regulation of 3-MI production, which hinders efforts to control the odorant. While currently there is not a 3-MI producer isolated from animal waste, isolation of a 3-MI producing bacterium from swine waste lagoon would allow relevant studies of the decomposition process, possibly enabling the reduction of 3-MI levels.

Clostridium scatalogenes was used as a model in designing the isolation scheme for a bacterium from swine waste lagoon. An experiment by Doerner *et al.* (2008) suggested that addition of tryptophan to growth media resulted in an increase in the production of 3-MI by *Clostridium scatalogenes*. In another study, *Clostridium scatalogenes* and lagoon enrichments were grown in media

supplemented with various electron acceptors. Iron III stimulated 3-MI production to levels greatly exceeding production of 3-MI by the bacteria in media without this supplement (Doerner *et al*. 2008). Additionally, *Clostridium scatologenes* is an endospore former, thus implying that heat treatment may help in isolation of a bacterium. All of these factors were taken into consideration when designing the 3-MI enrichment strategy presented here.

Materials and Methods

Media Preparation

Samples were grown in two different types of media, tryptone-yeast extract (TY), a nutrient-rich, complex medium, and semi-defined (SD) with 1mM L-tryptophan, a media with limited growth components. TY media (1L) (Holdeman *et al.* 1977) was prepared by combining 20 g tryptone, 10 g yeast extract, 50 mL each of mineral solutions 1 (6 g K_2HPO_4 in 1000 mL nanopure H_2O) and 2 (12 g NaCl, 12 g $(NH_4)_2SO_4$, 6 g KH_2PO4, 1.2 g $CaCl_2 \cdot 2\ H_2O$, 2.5 g $MgSO_4 \cdot 7\ H_2O$ in 1000 mL nanopure H_2O) (Bryant and Burkey 1953), 4 mL (200mg/100mL) resazurin, 1 mL (25mg/100mL) hemin solution, and 4 µL (16 µM) vitamin K1 in 950 mL nanopure water. SD media (1L) was prepared by combining 1.8 g glucose, 50 mL each of mineral solutions 1 and 2 (Bryant and Burkey 1953), 1 mL Pfenning trace minerals (0.1 g $ZnSO_4 \cdot H_2O$, 0.03 g $MnCl_2 \cdot H_2O$, 0.3 g H_3BO_3, 0.2 g $CoCl_2 \cdot 6\ H_2O$, 0.01 g $CuCl_2 \cdot 2\ H_2O$, 0.2 g $NiCl_2 \cdot 6\ H_2O$, 0.03 Na_2MoO_4, 1.5 g $FeCl_2 \cdot 4\ H_2O$, 0.01 g Na_2SeO_3 in 1000 mL nanopure H_2O), 2 g yeast extract, 5 g $[NH_4]_2SO_4$, 4 mL (200mg/100mL) resazurin, 1 mL

(25mg/100mL) hemin solution, 2.7 g sodium bicarbonate, and 10 µL (16 µM) vitamin K1 in 950 mL nanopure water. Each independent solution was boiled under bubbling CO_2 gas for 10-15 minutes, then rapidly cooled in an ice bath, after which 1 g cysteine-HCl was added. When the media was anaerobic, as indicated by resazurin changing from blue to colorless, the pH of the media was adjusted to approximately 7.2 (to obtain a final pH of 6.5-7.0, as autoclaving reduced the pH) using freshly-prepared 12 M NaOH. The flask of media was capped and moved into the anaerobic hood. The anaerobic hood (Coy Laboratory Products, Inc. Grass Lake, MI) was maintained with an atmosphere of 95% carbon dioxide : 5% dihydrogen. Media was adjusted to 1 L (1000 mL) using sterile anaerobic water. The media was then dispensed into 18 x 150 mm Balch-type tubes (Belco Glass, Inc., Vineland, NJ), sealed with rubber stoppers and aluminum crimps, and then autoclaved for 15 minutes at 121-123°C and 15 psi. Stock solutions (lagoon slurry, rumen fluid, IAA, etc) for additions to medium were filter sterilized by passing through a sterile 0.22 µm filter into a sterile anaerobic serum bottle or Balch tube. When different carbohydrate sources were tested, SD media was prepared with glucose omitted.

For anaerobic plates, tryptone-yeast extract medium was prepared using the recipe described above, and 15 g agar was added to 1 L of media. Media was autoclaved for 15 minutes at 121-123°C and 15 psi then cooled to 55°C. The flask of media was capped and moved into the anaerobic hood, where plates were poured and stored at room temperature until used.

Sample Collection and Inoculation Conditions

The lagoon sample was obtained from the anaerobic sediment of WKU's primary swine waste lagoon and homogenized in a Waring blender for 5 minutes on maximum speed, then injected into four anaerobic media mixtures: TY, TY + Fe(III), SD + Trp, and SD + Trp + Fe(III). Both TY and SD were prepared with and without 10 mM Fe(III). Additionally, 10 mM Trp was added to every tube of SD. Each inoculation was serially diluted to 10^{-10} in each of the four conditions, and duplicates of each condition were performed and incubated at room temperature. The greatest dilution which exhibited 3-MI production was chosen for continued culturing; these selected cultures were then subdivided. Four milliliters of culture were continued in media, while the two remaining milliliters were subjected to a heat treatment. For heat treatment, two milliliters of sample were transferred to a sterile glass screwcap tube and heated at 70°C in a water bath for 30 minutes. Half a milliliter of sample was then injected into both TY + Fe(III) and SD + Trp + Fe(III) growth media for continued culturing; duplicates of each condition were run.

All cultures were transferred to fresh media every 2 weeks and tested for 3-MI. Individual colonies were isolated by plating for isolation on TY plates in anaerobic hood. Samples were plated at undiluted, 10^{-2}, and 10^{-4} dilutions. Resulting colonies were inoculated into TY media and screened for 3-MI production. Strains positive for 3-MI were streaked for isolation three times from pure colonies, and the strain with the highest level of 3-MI production was chosen for further study. When purified colonies were inoculated into a new

condition, they were transferred at least three times in this media condition to adapt the culture before testing. All conditions which demonstrated positive results were run in triplicate to verify 3-MI production.

Sample Preparation for HPLC Analysis

Two methods were utilized to extract 3-MI from cultures. In the first technique, a 1 mL sample was withdrawn from the culture into a 1.5 mL sterile Eppendorf tube. Samples were centrifuged at 13,000 rpm and 4°C for 10 minutes. One half a milliliter of supernatant was pipeted into a fresh Eppendorf tube, mixed with 0.5 mL acetonitrile, and incubated at -20°C for at least 30 minutes. Samples were then centrifuged at 13,000 rpm and 4°C for 5 minutes to remove any remaining particulates. All liquid in each Eppendorf tube was drawn up in 1 mL syringes, which were then attached to 25 mm 0.45 µm syringe filters. The liquid was injected through the filters into 1 mL ambershell vials and stored at -20°C until analyzed.

The second method was adopted for an increase in ease and efficiency of the extraction. 800 µL of each sample was removed to a sterile 1.5 mL Eppendorf tube. Tubes were centrifuged for two minutes at 13,000 rpm and 4°C. Thermo Hypersep C18 SPE columns were attached to a Promega vacuum manifold and equilibrated with 2-1 mL washings of HPLC grade methanol followed by 2-1 mL washings of HPLC grade water. Next, 500 µL of sample was injected into the columns. Columns were washed twice with 1 mL HPLC grade water. The columns were then placed in disposable 16X100 mm glass tubes using plastic adapters which suspended the columns at the top of the glass

tubes. One milliliter of acetonitrile was added to each column, and the columns were centrifuged for two minutes at 1,000 rpm and 4°C. Eluants were transferred to 1 mL ambershell vials and stored at -20°C until analyzed.

Screening for 3-MI by HPLC

For 3-MI detection, samples were analyzed by High Pressure Liquid Chromatography (HPLC) using a C-18 reverse phase column. Analytes were desorbed from the column with an increasing acetonitrile gradient. The first mobile phase consisted of 50 mM ammonium acetate (pH 6.0) with 10% acetonitrile, and the second mobile phase was 100% acetonitrile. Samples were detected using a fluorescence detector set at excitation wavelength of 275 nm and an emission wavelength of 348 nm; these wavelengths were found to be optimal for 3-MI detection by Doerner, *et al.* (2008). The threshold of detection was 0.1 ng. Samples were compared against authentic 3-MI standards.

Analysis of Isolate

Gram staining was performed with a Becton Dickinson and Company Gram Stain kit as described by the manufacturer. The 16S-ribosomal DNA was sequenced by PCR amplification using primers 27F – 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R – 5'-GGYTACCTTGTTACGACTT-3' and compared using the Ribosomal Database Project, Release 10 (Cole *et al.* 2008). Bacterial 16S rDNA was amplified from 2 µL of pure culture using Econotaq Plus 2x Master Mix and 1 µL of each 100 µM primer in a 50 µL reaction. DNA was denatured at 94°C for two minutes, after which 30 cycles of 94°C denaturation for 15 seconds, 52°C annealing for 15

seconds and 72°C extension for 1.5 minutes were performed. A final extension of 72°C for 10 minutes finished the reaction.

Results

Initial Inoculation

The initial inoculation of lagoon slurry was injected into four media mixtures: SD+Trp, SD+Trp+Fe(III), TY, and TY+Fe(III). Each was performed in duplicate and serially diluted to 10^{-10}, as shown in Figure 2. Screening of all the

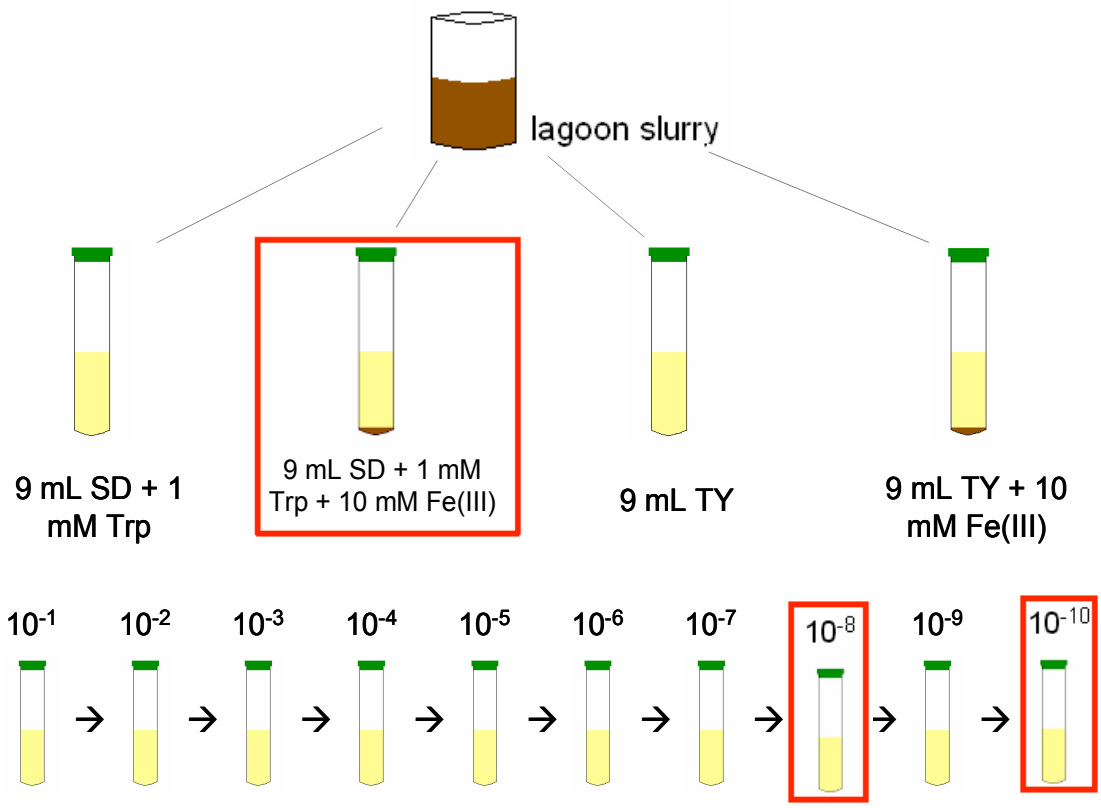

Figure 2. Initial inoculation of lagoon slurry sample into 4 media mixtures, each serially diluted to 10^{-10}. SD = Semi-Defined media; TY = Tryptone-Yeast extract media. The red boxes indicate the media type and the dilution selected for continued study.

samples for 3-MI produced the results displayed in Table 1. SD with Trp and

Fe(III) of dilutions 10^{-8} (Trial 1) and 10^{-10} (Trial 2) were chosen to continue transferring.

Table 1. 3-MI Production from Blended Swine Lagoon Slurry Serial Dilutions*

Condition	3-MI (Trial 1)	3-MI (Trial 2)	Condition	3-MI (Trial 1)	3-MI (Trial 2)
SD+Trp 10^{-1}	+	+	TY 10^{-1}	+	+
SD+Trp 10^{-2}	-	+	TY 10^{-2}	+	+
SD+Trp 10^{-3}	+	+	TY 10^{-3}	+	+
SD+Trp 10^{-4}	-	-	TY 10^{-4}	+	+
SD+Trp 10^{-5}	-	+	TY 10^{-5}	+	+
SD+Trp 10^{-6}	-	+	TY 10^{-6}	+	+
SD+Trp 10^{-7}	-	-	TY 10^{-7}	+	+
SD+Trp 10^{-8}	-	-	TY 10^{-8}	+	+
SD+Trp 10^{-9}	-	+	TY 10^{-9}	+	-
SD+Trp 10^{-10}	-	+	TY 10^{-10}	-	+
SD+Trp +Fe 10^{-1}	+	+	TY+Fe 10^{-1}	+	+
SD+Trp +Fe 10^{-2}	+	+	TY+Fe 10^{-2}	+	+
SD+Trp +Fe 10^{-3}	+	+	TY+Fe 10^{-3}	+	+
SD+Trp +Fe 10^{-4}	+	+	TY+Fe 10^{-4}	+	+
SD+Trp +Fe 10^{-5}	+	+	TY+Fe 10^{-5}	+	+
SD+Trp +Fe 10^{-6}	+	+	TY+Fe 10^{-6}	+	+
SD+Trp +Fe 10^{-7}	-	+	TY+Fe 10^{-7}	+	+
SD+Trp +Fe 10^{-8}	+	-	TY+Fe 10^{-8}	-	+
SD+Trp +Fe 10^{-9}	-	-	TY+Fe 10^{-9}	-	-
SD+Trp +Fe 10^{-10}	-	+	TY+Fe 10^{-10}	-	-

*(+) indicates a 3-MI presence in the sample. (-) indicates the absence of 3-MI in a sample.

Heat Treatment

The samples chosen from the serial dilutions were split. Four milliliters of each were continued in both SD+Trp+Fe(III) and TY+Fe(III). The remaining two milliliters were heat treated, then also inoculated into SD+Trp+Fe(III) and TY+Fe(III). (Figure 3) Duplicates were made of each condition. Eight of these 16 samples were positive for 3-MI and were transferred into the same media

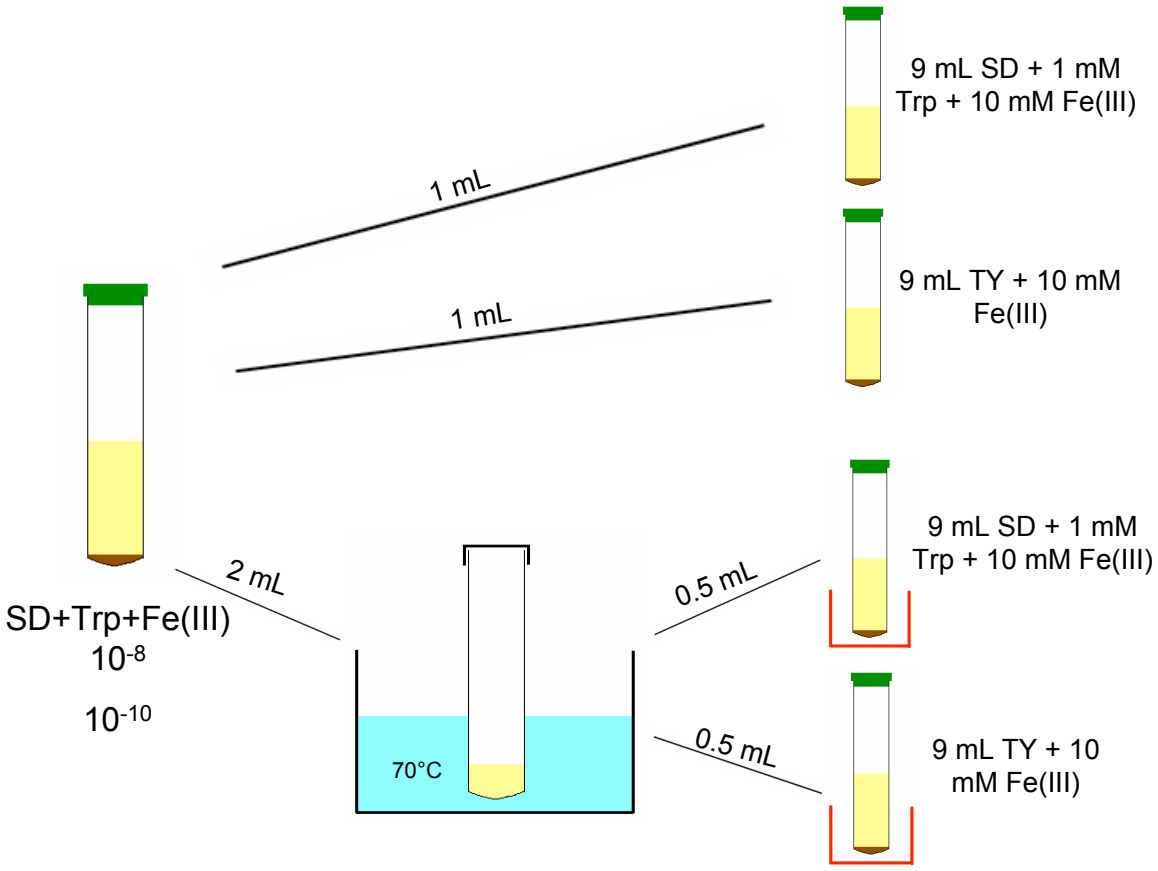

Figure 3. Transfer scheme for the 10^{-8} and 10^{-10} dilutions of SD+Trp+Fe(III) via regular transfer and heat treatment. Duplicates were made of each condition.

(SD continued in SD; TY continued in TY). Additionally, samples in SD+Trp+Fe(III) were also transferred into TY+Fe(III) to provide additional nutrients. The samples producing the 10 highest amounts of 3-MI were chosen to plate out in order to isolate a 3-MI producer in pure culture.

Plating for Isolation

Of those samples chosen for further study, five had been heat treated, and five samples were from non-heat treated conditions, as illustrated in Figure 4. Each was plated on TY from undiluted culture as well as 10^{-2} and 10^{-4}

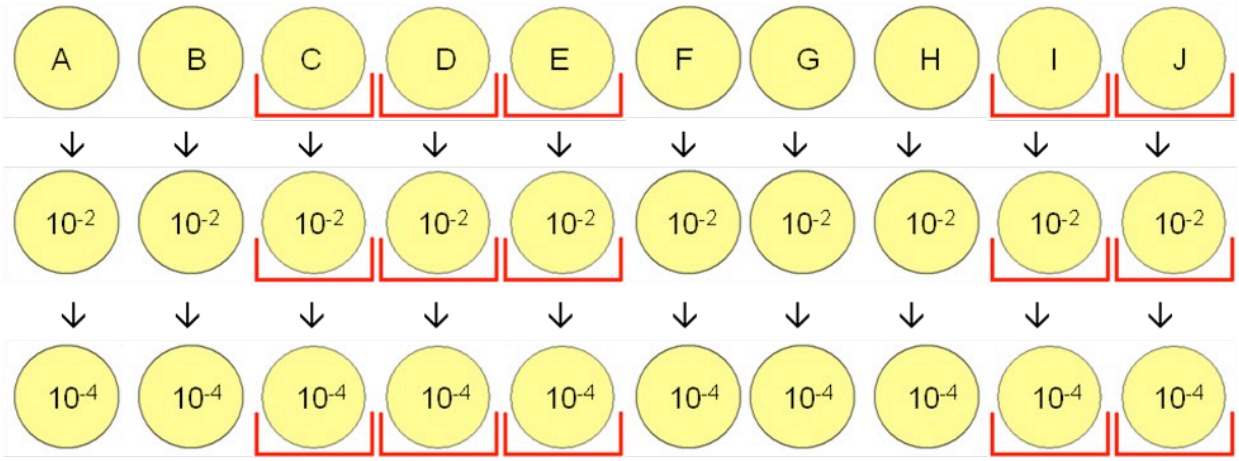

Figure 4. Plating scheme for ten 3-MI producing cultures. Red underlining indicates sample was heat treated.

dilutions, and incubated in the anaerobic hood at room temperature. Only one of these plates, an undiluted, heat-treated culture, showed growth; 10 colonies were produced. Each colony was inoculated into TY+Fe(III) and grown at room temperature; five of the ten were positive for 3-MI. The five 3-MI-producing samples were streaked for isolation three times on TY plates, and two colonies from each plate were inoculated into TY+Fe(III). 3-MI levels were measured, and the isolate with the highest production was chosen for detailed studies.

Strain Identification

The 16S ribosomal DNA was amplified and sequenced (Figure 5) then compared to the Ribosomal Database Project (Cole *et al.* 2008). Results indicated a 99-100% sequence similarity to *Enterococcus casseliflavus* (Figure 6). Also, the lagoon isolate is a Gram positive cocci, and usually appears as singular, ovoid cells or in short chains (Figure 7). The bacterium was designated *Enterococcus sp.* 4812.

AGAGTTTGATCATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGC
TTTTTCTTTCACCGGAGCTTGCTCCACCGAAAGAAAAAGAGTGGCGAACGGGTGAGTAACACG
TGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACACT
ATTTTCCGCATGGAAGAAAGTTGAAAGGCGCTTTTGCGTCACTGATGGATGGACCCGCGGTGC
ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGT
GATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT
CTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATC
GTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAAAATGTTCATCCCTTGACGGTATCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTT
GTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCC
CGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGA
ATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTC
TCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCA
GCAAACGCATTAAGCACTCCGCCTGGGGAGTACGACGCAAGGTTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACC
AGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGG
TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCG
GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTA
CAATGGGAAGTACAACGAGTTGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCA
GTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAG
CACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTG
TAACACCCGAAGTCGGTGAGGTAACCTTTT

Figure 5. 16S-ribosomal sequence for *Enterococcus sp. 4812*. Primers: 27F and 1492R

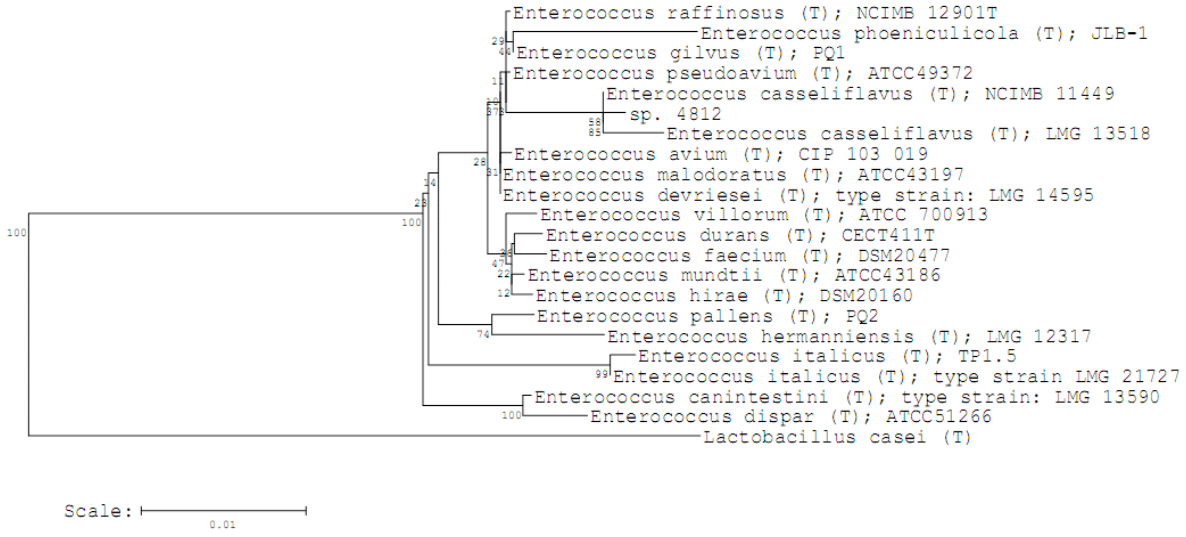

Figure 6. Phylogenetic tree generated from 16S-ribosomal sequencing with the Ribosomal Database Project

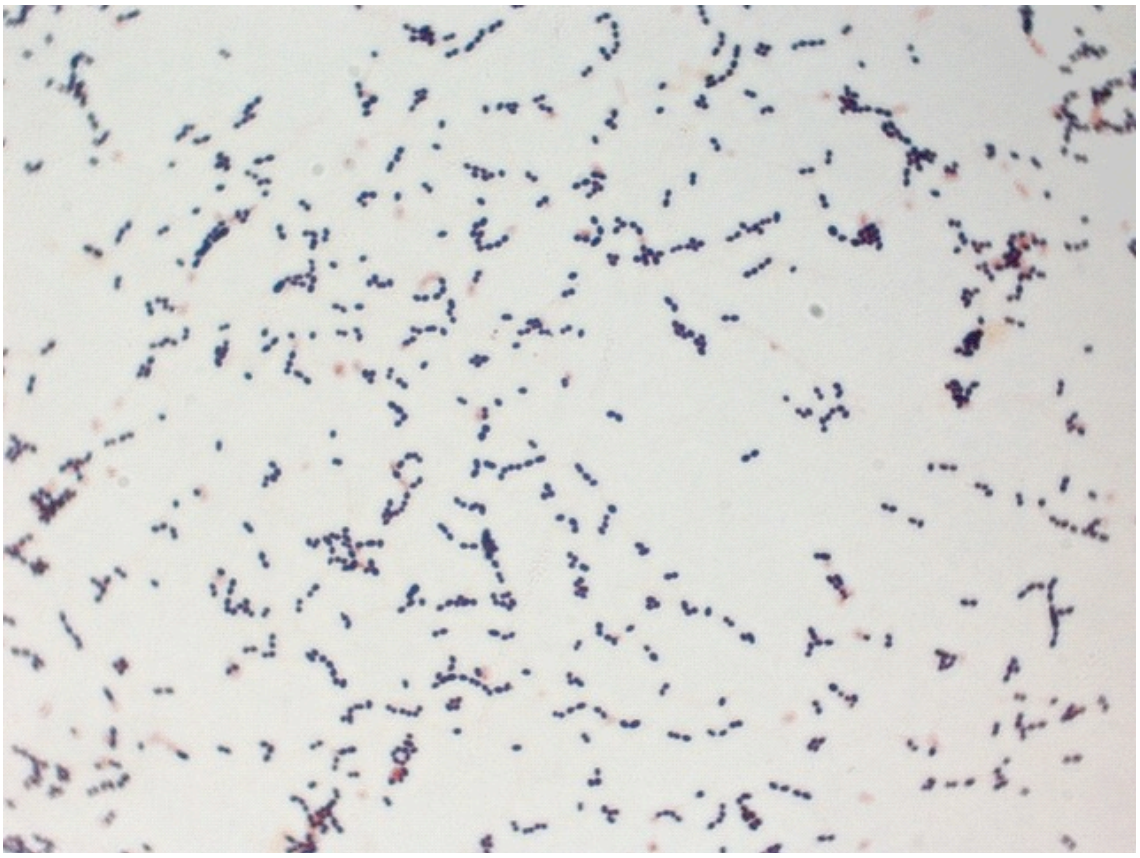

Figure 7. Gram stain of *Enterococcus sp. 4812*.

Regaining 3-MI Production

Although the cells continue to grow well, successive culturing resulted in a loss of 3-MI production by *Enterococcus.sp.* 4812. To regain the production of 3-MI, a variety of growth conditions were tested; results are displayed in Table 2. Some conditions produced 3-MI, but production was not maintained in sequential transfers. These sporadic positives resulted in samples that had additions of sterilized lagoon slurry or rumen fluid, although not all samples with lagoon slurry or rumen fluid additions produced positive results.

Table 2. Growth Conditions Tested with Isolated *Enterococcus sp. 4812**

Condition (all grown at RT unless otherwise noted)	**3-MI**
SD	-
SD + 1 mM Trp	-
SD + 100 µM 3-MI	-
SD + 1 mM Trp + 10 mM Fe(III)	-
SD + 1 mM Trp + 10 mM Fe(III) + 100 µM 3-MI	-
SD + 10% lagoon slurry	~
SD + 10% lagoon slurry (30°C)	-
SD + 10% lagoon slurry (37°C)	-
SD + 20% lagoon slurry	-
SD + 20% lagoon slurry (30°C)	-
SD + 20% lagoon slurry (37°C)	-
SD + 10% lagoon slurry + 1 mM Trp	-
SD + 10% lagoon slurry + 1 mM Trp + 10 mM Fe(III)	-
SD + 10% lagoon slurry + 1 mM Trp + 10 mM Fe(III) (30°C)	-
SD + 10% lagoon slurry + 1 mM Trp + 10 mM Fe(III) (37°C)	~
SD + 10% lagoon slurry + 10 µM 3-MI	-
SD + 10% rumen fluid	~
SD + 10% rumen fluid + 1 mM Trp	-
SD + Sigma Vitamins	-
SD + Sigma Vitamins + 1 mM Trp + 10 mM Fe(III)	-
all above SDs had glucose as their carb source	
TY	-
TY + 10 mM Fe(III)	-
TY + 10 mM Fe(III) (30°C)	-
TY + 10 mM Fe(III) (37°C)	-
TY + 10 mM Fe(III) + 1 mM Trp	-
TY + 10% lagoon slurry	-
TY + 10% lagoon slurry + 1 mM Trp	-
TY + 10% rumen fluid	~
TY + 10% rumen fluid (30°C)	-
TY + 10% rumen fluid (37°C)	-
TY + 20% rumen fluid	~
TY + 20% rumen fluid (30°C)	-
TY + 20% rumen fluid (37°C)	-
TY + 10% rumen fluid + 1 mM Trp	-
TY + 10% rumen fluid + 100 µM 3-MI	-
SD + 10 mM sucrose	-
SD + 10 mM sucrose + 1 mM IAA	-
SD + 10 mM lactose	-
SD + 10 mM lactose + 1 mM IAA	~
SD + 20 mM glycerol	(no growth)
SD + 20 mM glycerol + 1 mM IAA	(no growth)
SD + 10 mM mannitol	-
SD + 10 mM mannitol + 1 mM IAA	-
SD(glucose) + 1 mM IAA	-
SD + Sigma Vitamins + 1 mM IAA	-
SD + 1 mM IAA + 10 mM Fe(III)	-
TY + 1 mM IAA	-

*3-MI results: (-) indicates no 3-MI was detected; (~) indicates 3-MI was detected in an isolated incident, but production was not consistently maintained by the sample.

Discussion

The goal of this project was to isolate a 3-MI producing bacterium from swine waste lagoon. Methods such as serial dilutions and heat treatment were utilized. From the initial inoculation serial dilutions, both media types (SD and TY) produced positive 3-MI results to 10^{-10}. A culture in SD with Fe(III) was chosen for further analysis since it was grown in a more defined medium. TY is a richer, more complex media, which could have facilitated the growth of many kinds of bacteria. Cultures were grown in TY in case SD failed to grow a 3-MI producer. SD has more limited nutrient resources, supporting growth of fewer kinds of bacteria. Detecting 3-MI production in SD could indicate that a 3-MI producer had out-competed other bacteria, and likely, more non-target bacteria had been eliminated.

Clostridia as a genus are characterized as endospore-formers, thus heat treatment during the isolation process was attempted to enrich for an endospore-producing bacteria and possibly a 3-MI producer. Surprisingly, the isolated strain contained no endospores, and thus this vegetative cell appears to be heat resistant. This is a known characteristic of the *Enterococcus* genus (Holt *et al.* 2004).

3-MI production was lost by *Enterococcus sp.* 4812 in axenic culture after transferring over two months. Many growth conditions were tested to account for a possible lacking nutritional component. A vitamin mixture (#R7256 Sigma-Aldrich Co. St. Louis, MO) was added in some SD media to add additional nutrients for the bacteria. No effect on 3-MI production was observed. It was

possible that the bacterium was missing a component possibly present in its natural environment that stimulated 3-MI production. To test this hypothesis, sterilized lagoon slurry and rumen fluid were investigated as possible additions. A few independent positive 3-MI results were observed, but none lasted through subsequent transfers. On the possibility that 3-MI itself stimulated 3-MI production, 100 μM 3-MI was added to media, with no effect observed. Tests of *Enterococcus sp.* 4812 growth at room temperature, 30°C, and 37°C showed no effect by temperature on 3-MI production, although all temperatures supported growth.

Another possible way to restart 3-MI production involved substituting different carbohydrate sources into the SD media in place of glucose. Sucrose, lactose, and mannitol all supported growth, but glycerol did not. Still, none of the conditions restored 3-MI production. The addition of L-tryptophan resulted in increased 3-MI levels in cultures of *C. scatologenes*, and the addition of indoleacetic acid gave elevated 3-MI levels in *Lactobacillus sp.* 11201. Tryptophan was added to media throughout the isolation process to enrich for a 3-MI producer, but no effect was observed. When IAA was added to media, 3-MI levels did not exceed the background levels.

An experiment by Doerner *et al.* (2008) suggested that the addition of the electron acceptor Fe(III) to *C. scatologenes* and lagoon enrichments will increase 3-MI levels. Fe(III) was used as an isolation condition on the possibility that it would provide a stimulatory effect for 3-MI producers. However, after the isolation of *Enterococcus sp.* 4812 in pure culture, 3-MI production became

unstable. The effects of iron were therefore not measured as the results would have been unreliable.

Another consideration derived from this experiment concerns the isolation process of *Enterococcus sp.* 4812. Several of the enrichment schemes were based on studies performed on *Clostridium scatalogenes,* particularly the heat treatment and iron enrichment. Why was a *Clostridium* species not isolated? The isolation of an *Enterococcus* species instead of a *Clostridrium* was surprising. Overall, this simply points out that much more work needs to be done to answer this question and elucidate the physiological relationship between *Clostridia* species and *Enterococcus* species in lagoon samples.

Further examination needs to be done of the nutritional components of *Enterococcus sp.* 4812 to enlighten upon what the species requires for 3-MI production in lagoons. Work by Cook *et al.* showed that 3-MI is produced by microbial populations in lagoon slurry enrichments (2007). Possibly, a microbial consortium is required with bacteria of another type involving the presence of another molecule or some kind of cell signaling.

In a study performed by Cotta, Whitehead and Zeltwanger (2003), strains of microorganisms were identified from swine fecal and lagoon slurry samples and physiologically characterized. The most numerous isolates obtained from lagoon slurry samples were most similar to *Clostridium coccoides* and *Enterococcus* groups, as defined by 16S rDNA sequence analysis. Although 3-MI levels or other odorants produced by these cultures were not examined, it is clear that *Enterococci* make up a large portion of the bacterial populations in pit

lagoons. Therefore further study is warranted. A notable result from the study by Cotta *et al.* (2003) is that the distribution of species found in fecal samples was much broader than the distribution in lagoon slurry, likely due to the specific, selective environmental conditions present in the two different ecosystems. In fact, the most notable difference between the two populations, as illuminated by the authors, was the large number of *Enterococcus* group organisms found in lagoon samples whereas they were nearly absent in fecal samples. This could be due simply to having favorable conditions for *Enterococcus* in swine waste lagoons, while they were outcompeted within the feces. Further study of the physiology of *Enterococcus* should expound on these findings.

In addition to the above mentioned considerations, future work for *Enterococcus sp.* 4812 includes a study of the effects of Fe(III), both concentration and type of Fe(III) dependency. *Enterococcus spp.* constitute a large portion of lagoon bacterial populations, lending it a measure of importance in the study of lagoon odorants. The process of regaining 3-MI production in this strain could give insight on cell metabolism, as the method used to restart production would elucidate related factors to 3-MI production. This isolated strain may be useful as a model system to study 3-MI production in swine waste lagoons. The strain would also enable researchers to determine the genes involved in 3-MI production, estimate levels of this organism in lagoons, and gain insight on the biochemistry of the organism.

Literature Cited

Bryant, M.P. and L.A. Burkey. (1953) Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *J Dairy Sci* 36:205-217.

Chung, K. T., G. M. Anderson, and G. E. Fulk. (1975) Formation of indoleacetic acid by intestinal anaerobes. *J Bacteriol* 124:573-5.

Cotta, M.A., T.R. Whitehead, and R.L. Zeltwanger. (2003) Isolation, characterization and comparison of bacteria from swine faeces and manure storage pits. *Environmental Microbiology* 5(9): 737-745.

Cole, J.R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R.J. Farris, A.S. Kulam-Syed-Mohideen, D.M. McGarrell, T. Marsh, G.M. Garrity, and J.M. Tiedje. (2008) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research*. 37:D141-D145.

Cook, K. L., M. J. Rothrock Jr., J. H. Loughrin, and K. C. Doerner. (2007) Characterization of skatole-producing microbial populations in enriched swine lagoon slurry. *Microbial Ecology* 60(2007): 329-340.

Deslandes, B., C. Gariépy, and A. Houde. (2001) Review of microbiological and biochemical effects of skatole on animal production. *Livestocks Production Science* 71(2001): 193-200.

Doerner, K.C., B.P. Mason, D. Kridelbaugh, and J. Loughrin. (2008) Fe(III) stimulates 3-methylindole and 4-methylphenol production in swine lagoon enrichments and *Clostridium scatologenes* ATCC 25775. *Lett Appl Microbiol* 48:118-124.

Doerner, K.C., K. Cook, and B. Mason. (2008) 3-Methylindole production is regulated in *Clostridium scatologenes* ATCC 25775. *Lett Appl Microbiol.* 48:125-132.

Holdeman, L.V., E.P. Cato, and W.E.C. Moore. (1977) *Anaerobe Laboratory Manual.* Blacksburg: Virginia Polytechnic Institute and State University

Holt, J.G., N.R. Krieg, P.H.A. Sneath, J.T. Staley, and S.T. Williams. (2004) *Bergey's Manual of Determinative Bacteriology, 9th ed.* Philadelphia, PA: Lippincott Williams and Wilkins.

Mackie R.I., P.G. Stroot, and V.H. Varel. (1998) Biochemical identification and biological origin of key odor components in livestock waste. *J Anim Sci.* 76:1331-1342.

Whitehead, T.R., N.P. Price, H.L. Drake, and M.A. Cotta. (2008) Catabolic pathway for the production of skatole and indoleacetic acid by the acetogen *Clostridium drakei, Clostridium sctologenes*, and swine manure. *Appl Environ Microbiol* 74:1950-1953.

Yokoyama, M.T. and J.R. Carlson. (1981) Production of skatole and *para*-cresol by a rumen *Lactobacillus sp*. *Applied and Environmental Microbiology* 41:71-76.

Yokoyama, M.T., J.R. Carlson, and L.V. Holdeman. (1977) Isolation and characteristics of a skatole-producing *Lactobacillus sp.* from the bovine rumen. *Amer Society for Microbiol* 34:837-842.